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# EFFECTS OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND ITS SIGNALING PATHWAY ON SENSORY NEURONAL ACTIVATION DURING COLITIS

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EFFECTS OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND ITS SIGNALING  
PATHWAY ON SENSORY NEURONAL ACTIVATION DURING COLITIS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of  
Science in Physiology and Biophysics at Virginia Commonwealth University.

by

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## Table of Contents

	Page
Acknowledgements .....	ii
List of Figures .....	iv
Abstract .....	vi
Chapter	
1    Introduction .....	8
Inflammatory Bowel Disease .....	8
Visceral Hypersensitivity in the Dorsal Root Ganglion .....	10
Neurotrophins .....	13
Brain-derived Neurotrophic Factor (BDNF) .....	15
BDNF Signaling Cascades .....	16
cAMP-Response Element Binding Protein (CREB) .....	18
Calcitonin-Gene Related Peptide .....	18
Role of BDNF in Colitis .....	19
2    Objective, Aims, and Research Design .....	21
3    Methods .....	23
4    Results .....	31
5    Discussion .....	66
List of References .....	70

## List of Figures

	Page
Figure 1: Projections of Spinal afferents to visceral organ and CNS .....	11
Figure 2: Major neurotrophins and their receptors .....	13
Figure 3: p75 <sup>NTR</sup> receptor binding and effects .....	14
Figure 4: Major signaling pathways of BDNF .....	17
Figure 5: Animal models used for tissue dissection. ....	27
Figure 6: Rat spinal cord and DRG.....	29
Figure 7: Changes in CGRP protein expression during colitis. ....	34
Figure 8: Changes in CGRP mRNA expression during colitis.....	36
Figure 9: Effects of BDNF on CGRP protein expression <i>in vitro</i> .....	38
Figure 10: Effects of PLC inhibitor on CGRP protein expression <i>in vitro</i> .....	40
Figure 11: Effects of MEK kinase inhibitor on CGRP protein expression <i>in vitro</i> .....	42
Figure 12: Effects of PI3K inhibitor on CGRP protein expression <i>in vitro</i> .....	44
Figure 13: PLC- $\gamma$ activation during colitis in rat DRG.....	46
Figure 14: Co-localization of CGRP and PLC- $\gamma$ . ....	48
Figure 15: PLC inhibitor treatment on CGRP protein expression in colitis .....	50
Figure 16: PLC- $\gamma$ during anti-BDNF treatment in colitis-induced rat DRG. ....	52
Figure 17 Co-localization of CGRP and p-Akt.....	54
Figure 18: PI3K inhibitor treatment on CGRP protein expression in colitis.....	56
Figure 19: Co-localization of CGRP and p-CREB.....	58

Figure 20: CREB phosphorylation during colitis .....	60
Figure 21: Variations in CREB phosphorylation <i>in vitro</i> .....	62
Figure 22: Upstream regulators of CREB phosphorylation <i>in vitro</i> .....	64

## Abstract

### EFFECTS OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND ITS SIGNALING PATHWAY ON SENSORY NEURONAL ACTIVATION DURING COLITIS

By Fiza Hashmi, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of  
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Major Director: Dr. Liya Qiao, PhD  
Associate Professor  
VCU School of Medicine  
Physiology and Biophysics

Visceral hypersensitivity is the heightened response to sensory stimuli. Visceral sensations are transmitted through primary afferent neurons in the dorsal root ganglion (DRG) and the sensitization of the neural pathway leads to modification in spinal ascending and descending neurons. The aim of this investigation is to determine the effects of brain-derived neurotrophic factor (BDNF) and its signaling pathway on sensory neuronal activation during colitis. In order to evaluate this, levels of calcitonin-gene related



peptide (CGRP), a neuropeptide marker for nociceptive transmission, and phosphorylated cAMP-response element binding protein (pCREB), a molecular switch in neuronal plasticity, were studied in response to BDNF *in vivo* and *in vitro*. Colitis caused an increase in the levels of CGRP and pCREB in thoracolumbar DRG, which was attenuated by BDNF neutralizing antibody and PLC inhibitor, U73122, but not PI3K inhibitor, LY294002. BDNF-induced CGRP expression and CREB activation in DRG culture was also blocked by PLC inhibitor, U73122, but not PI3K inhibitor, LY294002, or MEK kinase inhibitor, PD98059. These results suggest a unique signaling pathway, i.e. the PLC- $\gamma$  pathway, is mediating BDNF action on sensory neuronal activation during colitis.

# Introduction

## 1.1 Inflammatory Bowel Disease

In 1859, Sir Samuel Wilks was the first to describe inflammatory bowel disease (IBD), specifically ulcerative colitis, in his account of his patient, Isabella Bankes. In this account, he wrote:

In the small intestine nothing remarkable was observed until the lower end of the ileum was reached, when at about three feet from its termination in the caecum, the mucous membrane commenced to exhibit an inflammatory response. In the caecum, inflammation of the most acute and violent character was observed...[11]

Wilks's diagnosis was later amended to Crohn's disease in the mid-1950s. This description, however, inaugurated documentation in the late 19<sup>th</sup> century of the two main forms of inflammatory bowel disease: Crohn's disease (CD) and ulcerative colitis (UC).

Today, the prevalence of these diseases is widespread, most commonly found in Europe and North America [4, 6]. In Europe, the reported incidence of CD is 322 per 100,000 persons and 505 per 100, 000 person for UC [7]. The prevalence in North America for CD is 319 per 100, 000 persons and 249 per 100,000 persons [7]. Four percent of IBD cases are not distinguishable between UC and CD; these cases are termed indeterminate colitis [2, 4, 10]. The incidence of IBD is similar between men and women, and it also does not differ significantly by race [7]. Increased risk of IBD rises with age, peaking at early adulthood [7]. The prevalence and incidence of UC and CD is increasing with time and in different regions of the world, which points towards its development as a global disease.

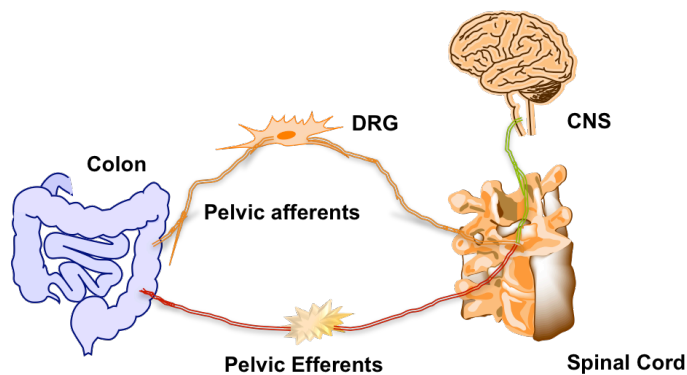
Ulcerative Colitis is a relapsing, debilitating inflammation focused on the colon. In the United States alone, 12 cases for every 100,000 persons is the annual prevalence for this disease

[1]. Endoscopic biopsy is the evaluative procedure used to confirm diagnosis [1]. The exact etiology of UC is yet unknown, although there are links to genetic and environmental factors [1, 2, 5]. Family occurrence reflects a genetic tendency to acquire the disease, but there is no gene or antigen present invariably in all patients [5]. Causes of UC have been linked to acute bacterial infections, yet no particular pathogens have been identified [8]. Patients demonstrate immunological abnormalities, suggested to be a result of inherited reactions to environmental elements [8]. Individuals with this disease exhibit symptoms such as diarrhea, excretion of pus, mucus, or both, and abdominal cramping [1, 5, 8]. The initiation of symptoms can be sudden or gradual and one-third of patients can present with extraintestinal symptoms such as arthritis, psoriasis, and uveitis [5, 8]. Regions of inflamed lesions are diffuse and superficial and also present in an uninterrupted fashion in the colon [1, 5]. As the disease progresses, there are flares that accompany periods of remission. Predicting the degree of inflammation and response to treatment is difficult because of the variability of the disease [1,5]. In young children, flares can be severe and unresponsive to medication [8]. Thus, much remains to be discovered in the pursuit of diagnosis and treatment of UC.

Crohn's disease is also a relapsing inflammatory disorder, but differs from UC in that it can affect the entire gastrointestinal tract [10]. It presents, however, mostly in the ileum and colon [3]. Compared to normal colon, there is intense swelling, inflammation, ulceration, and scarring. Symptoms of CD are diarrhea, abdominal pain, and fatigue. Strictures and fistulas, however, can form without any symptoms for years [2]. Inflamed lesions of mucosa can be intermittent with normal tissue and there are extraintestinal symptoms similar to those of UC [3]. It is evident that Crohn's disease can be a debilitating and life-threatening inflammatory bowel disorder.

Treatments of inflammatory bowel diseases can be divided into enzymatic and non-enzymatic approaches. These target potential causative factors such as reactive oxygen species, immune dysregulation, and pathogenic bacteria [12]. These treatments have found to be only slightly effective in inflammation and higher doses may alleviate symptoms of oxygen reactive disorder [12]. Further studies on possible novel treatments include enzymatic therapies, and use of eukaryotic and bacterial cell delivery systems.

## 1.2 Visceral Hypersensitivity in the Dorsal Root Ganglion



**Figure 1. Projections of Spinal afferents to visceral organ and central nervous system.** Primary afferent neurons are pseudounipolar cells that have axonal processes that project to the visceral organ and also have processes that project to the spinal cord from the DRG.

Chronic pains, such as those of inflammatory bowel disease, have multifaceted problems, which have no straightforward infectious, metabolic, or anatomical basis. These bowel disorders, despite variations in cause and symptoms,

all demonstrate lower abdominal pain.

Visceral hypersensitivity, or visceral

hyperalgesia, denotes a term used for expressing the enhanced sensation of visceral organs to stimuli [17]. Improper signaling in the viscera through the sensory reflex pathway can be a chief cause for abdominal pain [17]. Sensitization of the neural pathway, involving primary afferent neurons and spinal descending neurons, transmits visceral sensations to the supraspinal level and the maladaptation of descending pathways modulate spinal signaling (Figure 1) [14, 17].

Visceral organs in the thorax and abdomen, except for the pancreas, are innervated by both parasympathetic and sympathetic projections [13]. The primary sensory afferents are

pseudounipolar cells that have visceral points of connection as well as central axonal processes. In humans, the lumbar splanchnic nerve (LSN) and hypogastric nerve (HGN) from thoracolumbar outflows and the pelvic nerve (PN) from sacral outflows innervate the small and large intestine and the urogenital organs [13, 19]. The peripheral projections terminating on the visceral organ may innervate intramuscular arrays (IMAs), which are vagal mechanoreceptors on gastrointestinal smooth muscle [16]. This placement of visceral afferent neuron terminals allow for response to luminal and local chemical stimuli, as well as to mechanical (usually distending) stimuli. Projection on the other side of the afferent allows for central processes to enter the spinal cord through nodose ganglia or dorsal root ganglia (DRG), projecting mainly to lamina I and laminae V–VII [19]. The cell bodies of primary visceral afferent neurons are in the nodose ganglia, for vagal afferents, and the DRG, for spinal afferents, from which the information is carried further along the central nervous system in the spinal cord and brainstem [17, 19].

Primary visceral afferents have been shown to have a role in both acute and persistent pain contributing to the development of hyperalgesia. Visceral afferents have a high- and low-threshold response for visceral mechanosensation, which is primarily a result of organ distension [14]. “Silent,” or mechanically insensitive, visceral afferents seem to acquire spontaneous activity after tissue injury. It is not known the exact mechanism behind these afferent awakenings, whether it is due to experimental distention stimuli or if they are actually mechanically insensitive [14]. These afferents also could represent a group of chemonociceptors that become active after tissue insult and demonstrate polymodal sensitivity to different forms of sensory information [14]. The presence of these fibers in large quantities could suggest in increased torrent of afferent input, impacting visceral hyperalgesia [14].

Low-threshold visceral afferents have been shown to sensitize after experimental organ inflammation. Increased sensitization after inflammation signifies an increase in response magnitude, increase in spontaneous activity, and/or a decrease in response threshold [14, 19]. Biochemical mediators originating from immune and non-immune cells could factor into sensitization of the extrinsic visceral sensory innervation, molecules including amines, peptides, products of arachidonic acid metabolism, cytokines, neurotrophins, and reactive metabolites [19]. Thus, these mechanisms in response to inflammation could contribute to visceral hyperalgesia and enhanced sensitivity to normal contents.

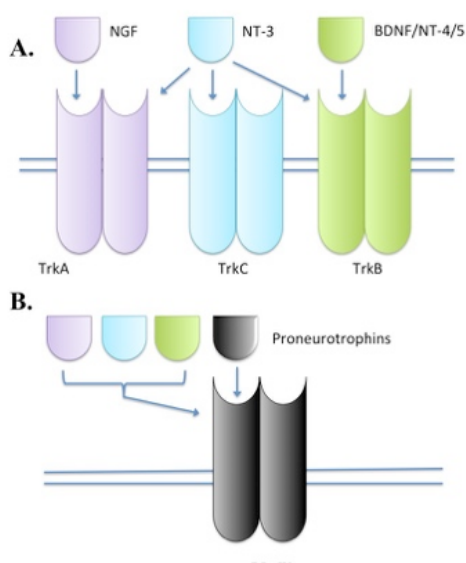
Visceral pain cannot be exactly mimicked in animal models because of its wide-ranging nature; however, experimental models have been developed in rats and mice. Instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) into rat colon provides an inflammatory model of colitis that closely parallels pain in human patients [18, 19]. TNBS is a hapten, a small molecule able to elicit an immune response by binding to antibodies when coupled to a carrier [15]. When rats are induced with TNBS, they present with ulcerative colitis-like symptoms [19]. Peak inflammation occurs 4 to 5 days after induction and remains for about a month [18]. They present with hypersensitivity to colonic distention and visceral hypersensitivity persists up to 16 weeks after the initial inflammation has been alleviated [18]. This model provides a foundation upon which to study the biochemical mediators involved in visceral hypersensitivity.

In summary, signals originating from the visceral organ are directed to extrinsic primary afferent sensory neurons in the DRG and/or the nodose ganglia. The information is processed and passed to higher processes in the central nervous system (CNS), after which descending nerves transfer excitatory or inhibitory signals that alter visceral organ function. The primary afferents demonstrate neuronal plasticity in response to biochemical stimuli and various

circumstances, which result in alteration of anatomical, biochemical, and electrophysiological characteristics of neurons.

### 1.3 Neurotrophins

The neurotrophin family is generally recognized to provide neuroprotection, synaptic plasticity, and neuronal cell development. Its members are vital mediators for neuronal activity



**Figure 2. Major neurotrophins and their receptors.** A. NGF Binds to TrkA, BDNF bind to TrkB and NT-3 binds with high affinity to TrkC, with low affinity binding to TrkA and TrkB. B. Proneurotrophins and neurotrophins also bind to p75<sup>NTR</sup>.

in both the central and peripheral nervous system.

There are four main mammalian neurotrophins: Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4/5 (NT-4/5). The receptors for each neurotrophin are tyrosine kinase A (TrkA) binding to NGF, TrkB binding to BDNF or NT-4, and TrkC binding to NT-3. Under certain conditions, NT-3 can also stimulate TrkA and TrkB (Figure 2) [17].

The general neurotrophin receptor (NTR) p75

bind to all the neurotrophins with low affinity when present alone in cells (Figure 3) [17, 26].

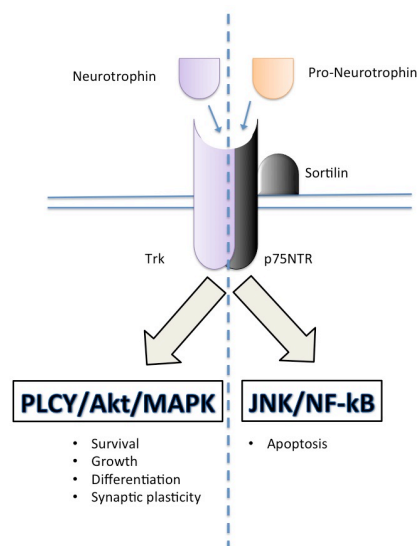
High-affinity binding between p75<sup>NTR</sup> and neurotrophins in the presence of the Trk receptor increases cell receptiveness. The response of neurotrophin/p75<sup>NTR</sup>/Trk complex is cell growth and survival signals (Figure 3). The contact between Trk receptors and p75<sup>NTR</sup> increases the affinity of Trk to various ligands that heighten cell growth and survival mechanisms through the stimulation of the phosphoinositide 3-kinase (PI3K)/Akt pathway or the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathway [17, 25, 26]. On

the other hand, proneurotrophins, the uncleaved precursors to neurotrophins, can bind to  $p75^{\text{NTR}}$  in the presence of sortilin and this complex leads to activation of apoptosis through the c-Jun N-terminal kinases (JNK), nuclear factor  $\kappa$ -B (NF $\kappa$ B), or Rho pathways (Figure 3) [17, 24]. These pathways, in turn, promote inflammatory responses. Another important pathway is the phospholipase C gamma (PLC $\gamma$ ) pathway, a signal cascade allows for calcium and sodium influx through ion channels that leads to long-term potentiation (LTP) and neuronal plasticity in CNS [17].

Thus, neurotrophins are important mediators that promote cell function through two primary means. One, they activate signal transduction cascades at the nerve terminal, and two, they are involved in the retrograde transport of signal mediators from nerve terminals to cellbodies [17]. Another form of transmission is anterograde transport where molecules are transported

from the cell body to axonal terminals. The primary afferent sensory fibers have neurons that signal complex nociceptive and non-nociceptive changes in visceral organs [26]. Neurotrophin expression and distribution

depend on different factors of cell type and development. For small-diameter primary sensory neurons of neural-crest origin of rats, NGF is essential for neuronal survival; however, as these animals mature, the cells become less dependent on NGF and change their expression of neurotrophic factor receptors [23]. BDNF interacts with these sensory neurons in maturity and it



**Figure 3.  $p75^{\text{NTR}}$  receptor binding and effects.** When NGF binds to the dimerization of TrkA and  $p75^{\text{NTR}}$ , signaling pathways promote synaptic plasticity, differentiation, and neuronal growth. However, when proneurotrophins, pro-NGF, in the presence of sortilin, binds to the TrkA/ $p75^{\text{NTR}}$  heterodimer, apoptosis ensues.



important for regulating mechanosensitivity of adapting touch mechanoreceptors [23], demonstrating BDNF's crucial role in sensory neuron development.

#### **1.4 Brain-Derived Neurotrophic Factor (BDNF)**

In 1982, through experimental investigation, it was shown that BDNF enhances the survival of cultured peripheral sensory neurons and enhances the survival of DRG and nodose ganglia neurons. BDNF is a basic protein initially isolated from pig brain and has a molecular weight of 12.4 kDa [30]. BDNF binds to TrkB and although most biological functions are attributed to the binding of full-length TrkB, some roles have been suggested for the truncated receptor as well [31]. BDNF and TrkB receptor have a wide distribution across the central nervous system. In the early stages of development, experimental transgenic mice have shown that BDNF plays a significant role in the cell survival of sensory neurons [31].

BDNF, like its other family members, can be retrogradely transported in sensory neurons [17, 23]. It has been shown that it can be transported to both of the peripheral nerve fibers and the spinal cord where it accumulates in the central terminals in the superficial laminae (I and II) [23]. The superficial laminae of the spinal cord are important sites for processing of nociceptive information. It was found that BDNF protein is localized mostly in the primary sensory nociceptors, in those that express TrkA and are sensitive to NGF [20]. In these neurons, BDNF is stored in dense core vesicles, possibly released in response to nociceptor activity [21].

BDNF has been shown to increase the frequency of excitatory post-synaptic currents in *Xenopus* cultures [27]. BDNF, in general, seems to heighten excitatory (glutamatergic) synapses and weaken inhibitory (GABAergic) synapses. This neurotrophin is an important neuromodulator in pain transduction. It is synthesized by the dorsal horn and in response to

inflammation injury in the peripheral nerves, BDNF is significantly upregulated [28]. BDNF sensitizes nociceptive afferents and causes hyperalgesia. It has been found that exogenous BDNF treatment increases nociceptive spinal reflex activity in rat spinal cord and that addition of BDNF inhibitors reduced the hyperalgesia. Kerr, et al. demonstrated that this spinal reflex was reduced when spinal cord was treated with BDNF-sequestering antibody TrkB-IgG [29]. This group also showed that endogenous BDNF affects behavioral nociceptive responses as well [29].

Conditional mouse knockouts deleting BDNF showed that null animals were healthy with no reduction in sensory neurons, but pain-related behaviors were significantly affected [32]. It was determined from these knockout mice that BDNF plays a crucial role in regulating inflammatory pain thresholds [32]. Studies have shown that MAP kinases ERK and p38 are important in the signal transduction cascade upregulating BDNF in these pain-related behaviors [33]. Increased phosphorylation of ERK in lamina I of the dorsal horn has been shown in neurons transmitting pain hypersensitivity. Regulation of these signaling cascades plays a crucial role in the expression of BDNF in pain-transducing neurons [34].

### **1.5 BDNF signaling cascades**

BDNF has significant roles in cell differentiation, growth, survival, neuronal branching, and strengthening synaptic function [35, 36]. Effects of BDNF are also coupled to LTP in the hippocampus through autophosphorylation of the TrkB receptor, activating the Ras/ERK, phosphatidylinositol 3-kinase/AKT, or PLC- $\gamma$  pathways (Figure 4) [37].

Activation of the Ras/ERK begins when a tyrosine residue on the Trk receptor is phosphorylated and the receptor then binds to adapter proteins, such as Shc, Grb2, and Sos [39]. GDP is exchanged for GTP and this leads to activation of the protein Ras, which in turn activated

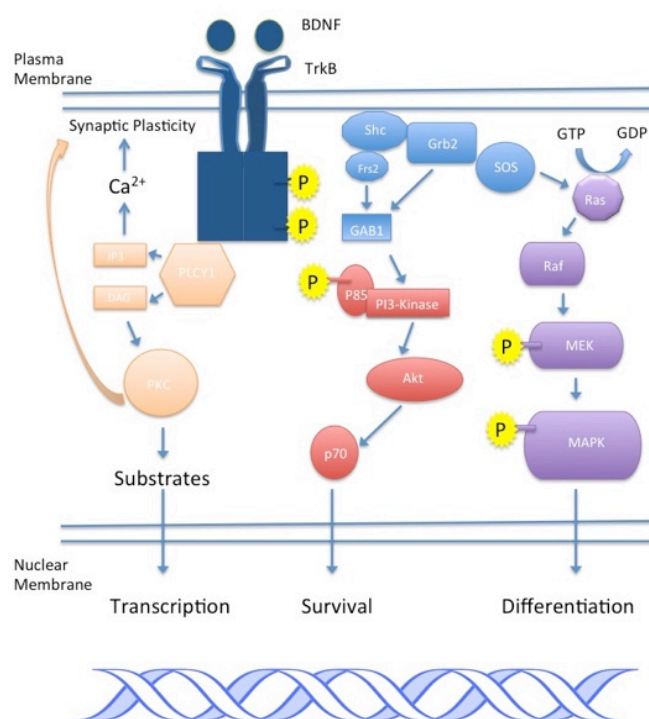
a number of kinases, such as Raf and MEK [40]. MEK then activates MAPK, which then is navigated to the nucleus where it can activate transcription factors such as Elk1 [40, 41]. Elk1 binds to the serum response factor, which is linked to the serum response element [42]. Ras can also phosphorylate and activate a number of kinases, which translocates to the nucleus and activates kinases [38, 42]. CREB then links to the cAMP-Ca response element (Ca-CRE) and

activates transcription of immediate early genes with the assistance of the SER complex [43].

The PLC- $\gamma$  pathway can interact with the Ras pathway to activate ERKs . TrkB can also couple with PLC- $\gamma$  to produce high frequency stimulation in association with LTP [44]. The postulated mechanism of action in the interaction of PLC- $\gamma$  with MEK is due to an inositol triphosphate (IP<sub>3</sub>)-mediated increase in intracellular calcium or diacylglycerol (DAG)-induced activate of protein kinase C (PKC) [45, 46].

Another mechanism linked to Trk

autophosphorylation is the PI-3 kinase pathway [45]. This pathway regulates many second messenger molecules, which in turn activate kinases, such as Akt, a protein that mediates most of the pathways effects [47]. The activation of Akt can lead to downstream effects, such as cell



**Figure 4. Major signaling pathways of BDNF.** Shown are the signaling cascades of the three major pathways of neurotrophin signaling: Ras/ERK (purple) , phosphatidylinositol 3-kinase/AKT (red), or PLC- $\gamma$  (orange). Each pathway activated by the binding of BDNF and the autophosphorylation of the Trk receptor can lead to activation of different transcription factors and alteration of gene transcription

survival, development, and proliferation. This pathway has been shown to activate the NF $\kappa$ B pathway that promotes cell survival and also downregulate the JNK/p38 pathway which leads to apoptosis (48, 49).

Thus, activation of Trk receptor through autophosphorylation can lead to multiple cellular responses and activation of many signaling molecules. Kinetics, strength of signal, and the DNA-binding affinity of certain factors all contribute to the downstream activity of the BDNF signaling [26]. Activation of certain promoters for transcription may require the concurrent presence of many different transcription factors [49]. Understanding the signaling pathways is important for the understanding of how pain behaviors are molecularly transmitted.

### **1.6 cAMP-Response Element Binding Protein (CREB)**

CREB is a transcription factor that regulates an estimated 4,000 genes in humans and has been found to be involved in the perpetuation of neuropathic pain, inflammation pain, and chronic muscle pain [64-66]. Furthermore, inflammation-induced nociceptive behavior has been shown to associate with the phosphorylation of CREB (pCREB) [67-68]. This transcription factor is shown to be extensively involved in synaptic plasticity and long-term potentiation [70]. Activation of CREB is complex and may involve many different signaling molecules. More than 300 stimuli are suggested to phosphorylate CREB through intensification of Ca<sup>2+</sup> pathways and activation of kinases that phosphorylate CREB on serine 133 [69]. CREB could serve as an important mediator between extracellular signaling and regulation in gene transcription to nociceptive pain transmission.

### **1.7 Calcitonin-Genes Related Peptide (CGRP)**

Calcitonin-Gene Related Peptide (CGRP) is a meaningful neuropeptide marker in the biochemical response to pain perception. It is a 37 amino acid peptide and there are two types isolated so far: CGRP- $\alpha$  (CGRP-I) and CGRP- $\beta$  (CGRP-II) [50]. CGRP expression has been implicated in many systems including learning and memory [58], opioid tolerance and addiction [59], and pain transduction in the PNS and CNS [60]. It is found in a variety of nociceptive hypersensitivity mechanisms. Zhang et al. found that CGRP- $\alpha$  knockout mice were unable to develop hyperalgesia after induction of knee inflammation. Studies in rats and mice have helped to determine that CGRP plays in an important nociceptive role in neurogenic plasticity during peripheral inflammation [52, 53]. CGRP is rich in small-diameter unmyelinated neurons and considerably less so in medium- to large-diameter neurons [50, 54]. After induction of an inflammatory response, CGRP is upregulated significantly in primary afferent sensory neurons [55].

Recent studies have shown that colitis-induced rat DRG have increased mRNA and protein CGRP expression in the L1 segment [61]. There appears to be differential expression of CGRP along different thoracolumbar DRG and this could be due to divergent projections of sensory afferent fibers [61]. L1, L2, L6, and S1 DRG segments in rats have been shown to possess colonic primary afferent fibers [61, 62]. Another study has shown that retrograde NGF-induced CGRP expression operates through the extracellular signal-related protein kinase (ERK) pathway leading to CREB activation, leading to nociceptive transmission and facilitation of the sensation of inflammatory pain [63]. There appears to be an NGF-CREB-CGRP signaling axis and understanding the mechanism of neurotrophic signal transduction in response to inflammatory pain is a considerable stride in tackling what is unknown about sensory hypersensitivity from visceral inflammation.

## **1.7 Summary**

Previous studies have shown that BDNF increases in lumbosacral DRG in response to inflammation. [71]. However, the mechanism through which BDNF regulates synaptic changes and neuropeptide expression in colitis is not heavily studied. Extending from these previous studies, the goal of this study is to understand how inflammation-induced increase in BDNF regulates sensory neuron activation.

## Objectives and Aims

### Objectives

The objective of this study is to characterize the role of BDNF in sensory neuronal activity by examining CREB phosphorylation and CGRP expression in sensory neurons during colonic inflammation and to evaluate the signaling pathways that regulate these events.

### Hypothesis

Colitis-induced sensory neuronal activation is mediated by endogenous BDNF through one or more signaling pathways.

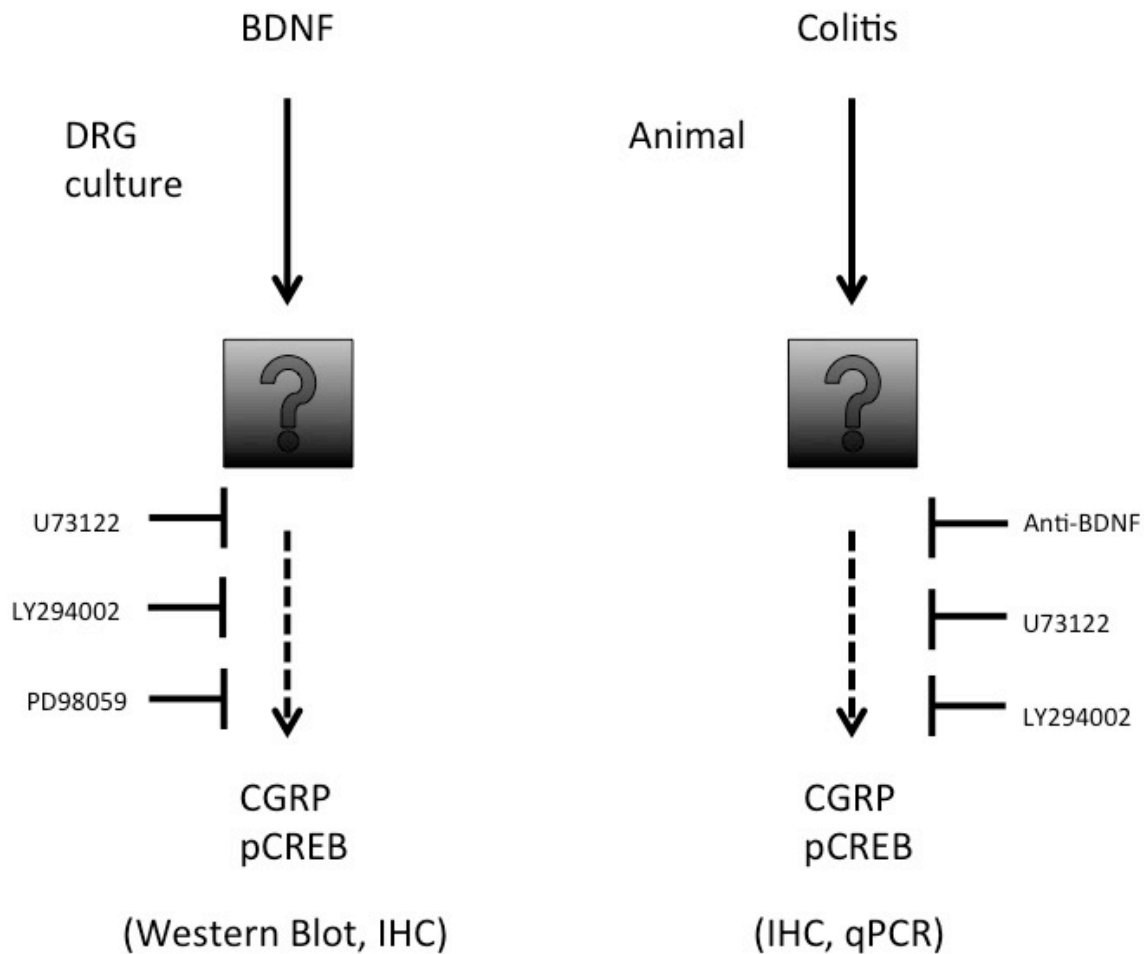
### Aims

Aim 1: Characterize the role of BDNF on CREB activation and CGRP expression in colitis.

Aim 2: Characterize the role of BDNF on CREB activation and CGRP expression in DRG culture.

Aim 3: Study the signaling pathways involved *in vitro* and *in vivo*.

## RESEARCH DESIGN



### BDNF In Vitro Analysis

L1, L2, L6, and S1 DRG (Figure 6) and ganglia were removed from naive animals. Pair-matched ganglia were treated with specific ligand or ligand with antagonist. Following incubation with the ligand or ligand with antagonist, the DRG were removed and analyzed with immunohistochemistry or western blot to view changes in protein expression.

### TNBS-induced Colitis and Dorsal Root Ganglia Dissection

TNBS was rectally administered in animals to induce colonic inflammation. Following either 3 or 7 days post-induction, rats were sacrificed and thoracolumbar DRG (L1, L2, L6, and S1) were removed. For antagonist studies, inhibitors were injected intraperitoneally following TNBS induction. Samples were prepared for analysis with RT-PCR or immunohistochemistry.



## Methods

### 3.1 Experimental Animals

Adult male rats weighing 150–200 g were used for all studies. All experimental protocols involving animal use were approved by the Institutional Animal Care and Use Committee at the Virginia Commonwealth University (IACUC # AM10315). Animal care was in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress or distress as well as to reduce the number of animals used.

### 3.2 Introduction of Colonic Inflammation in Rats with TNBS and Ganglia Dissection

Colonic inflammation was induced in rats by instillation of trinitrobenzene sulfonic acid (TNBS: 1.5 mL/kg of 60 mg/mL solution in 50% EtOH). Control animals received 50% EtOH. Rats were killed on day 3 or 7 following the induction of colitis. All injections were performed when rats were anesthetized under isoflurane (2%). Following sacrifice, rats were perfused with Krebs buffer and then 4% paraformaldehyde for use in immunohistochemistry. The L1, L2, L6, and S1 DRGs were removed for cryosectioning.

For BDNF *in vitro* studies, naïve animals were sacrificed and thoracolumbar DRG dissected. Membranous capsules surrounding the ganglia were then peeled away using fine forceps and DRG were then incubated in Dulbecco's Modified Eagle Medium (DMEM) for 2 hours. Inhibitor solutions were added one hour prior to BDNF stimulation. Following stimulation, DRG were prepared for western blot or immunohistochemistry. DRG were

processed for immunohistochemistry by transfer to 4% paraformaldehyde for 4-6 hours; and ganglia were then stored in 20% sucrose overnight.

### 3.3 Introduction of Antagonists in TNBS-induced Animals

Endogenous BDNF was blocked by 36 µg/kg of BDNF neutralizing antibody (anti-BDNF) through intravenous administration. In order to inhibit the PI3K pathway *in vivo*, an intraperitoneal injection of a PI3K inhibitor, LY294002 (Calbiochem), at a single dose of 50 µg/kg body weight was made 3 to 4 days after TNBS induction. To inhibit PLC pathway, animals were injected with U73122 at a dose of 1mg/kg body weight. Both U73122 and LY294002 solution was prepared by dissolving in DMSO as stock and then diluting in saline for injection. For *in vitro* studies, inhibitor concentrations of 5 µM for U73122, LY2944002, and PD98059 were added to DMEM solution one hour into ganglia incubation.

### 3.4 Quantitative Real-Time PCR (qPCR)

After animal sacrifice, L1 DRG was homogenized in lysis buffer using instructions according to RNAqueous kit (Ambion, TX) and total RNA was extracted. The total RNA in buffer then underwent reverse transcription using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI) under manufacturer's instruction. The cDNAs then were mixed with specific Taqman probes and PCR master mix (Applied Biosystems, ABI). qPCR was performed on StepOnePlus Real-Time PCR System (Applied Biosystems, ABI) with SYBR Green as the indicator. The target gene was calcitonin gene-related peptide (CGRP). β-Actin was used as an endogenous control. The changes in the target genes were normalized against the endogenous control and then were calculated to express fold changes using  $2^{-\Delta\Delta Ct}$ .

### 3.5 Immunohistochemistry

Ganglia were transferred from 20% sucrose and embedded in OCT cryostat medium (Tissue Tek). Each ganglion was then sectioned at a 20  $\mu$ m thickness and placed on gelatin-coated slides. Slides were then heated at 30°C for two hours. Slides were then incubated overnight with antibodies against CGRP (1:2000, Abcam), p-CREB (1:1000, Cell Signaling), p-Akt (1:500, Cell Signaling), and PLC- $\gamma$  (1:1500, Santa Cruz Biotechnology) at room temperature in 5% normal donkey serum and 0.3% Triton buffer. Following incubation, slides were washed with a 0.1M sodium phosphate buffer at a pH of 7.4 thrice for ten minutes each. Slides were then incubated with a fluorescent species-specific secondary antibody. Excess antibody was washed with the 0.1M sodium phosphate buffer thrice at ten minutes each cycle. Citifluor anti-fadent mounting medium (Electron microscopy Science) was then added to slides before cover-slipping. Slides were viewed with an Axiocam Carl Zeiss microscope and neurons exhibiting immunoreactivity greater than the observed background level were considered positively stained. The area of the DRG section was measured using spline tool, giving an area in micrometer squared, and number of positive neurons in the outlined area were counted. Numbers were then converted to millimeter squared Area and positive cells were totaled for one image and all images were averaged to give final number of positive cells per millimeter squared. For co-localizations, slides were double-stained according to the procedure above and photos were processed with Adobe Photoshop software.

### **3.6 Western Blot**

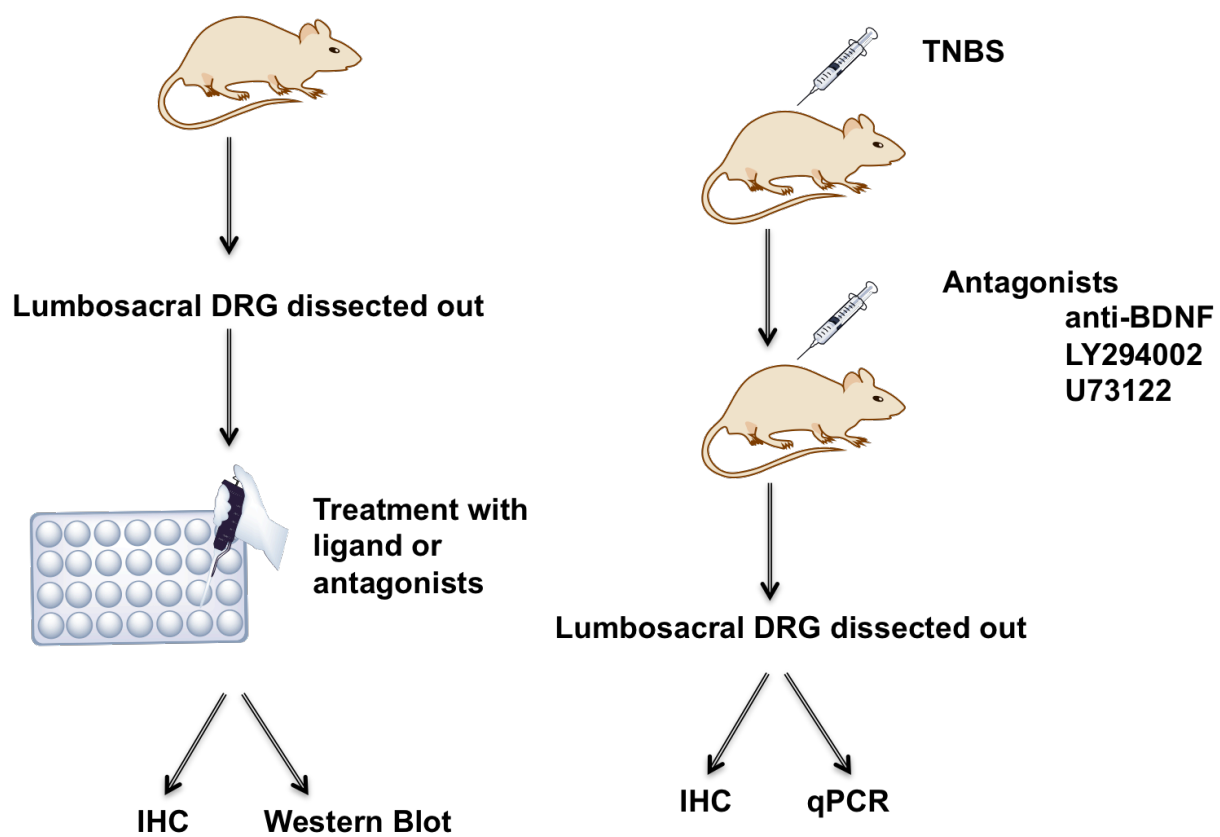
Ganglia were homogenized in T-Per solution (Pierce Biotechnology, Rockford, IL) with addition of protease and phosphatase inhibitor cocktails (Sigma). Protein extract was added to an equivalent amount of Laemmli Sample Buffer (Bio-Rad). Proteins were then separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane using Trans-Blot Turbo (Bio-Rad).

The membrane was blocked using 5% milk in Tris-buffered saline (TBS), washed thrice for ten minutes with TBS and 0.1% Tween-20 (TBST) solution, and incubated at 4°C with primary antibody against p-CREB (1:1000, Cell Signaling). Excess primary was washed thrice for ten minutes each cycle with TBST and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (1:2000, Cell Signaling) at room temperature. After washing, membranes were covered in West Femto Maximum Sensitivity Substrate (SuperSignal) to activate HRP enzymatic activity for observation of bands on x-ray film. For endogenous control, the same membrane was stripped and re-incubated with anti- $\beta$ -actin (1:2000, Sigma). The bands were visualized using ODYSSEY infrared imaging system (Li-cor Bioscience).

### **3.7 Statistical Analysis**

Results of each study are shown as the mean  $\pm$  SD. Control and experimental groups were compared by ANOVA with differences between means considered significant at  $p \leq 0.05$ . When two groups were compared, a *t*-test was used. GraphPad Prism 6 was used for analysis.

**Figure 5. Animal models used for tissue dissection. A.** TNBS-induced animals sacrificed after designated time and tissues collected for immunohistochemistry (IHC) and RT-PCR. **B.** Immunohistochemistry and western blot were used to analyze *in vitro* studies.



**Figure 6. Rat spinal cord and DRG.** DRG and spinal cord were dissected out after sacrifice and analyzed according to experimental design.





## Results

### 4.1 BDNF increases CGRP expression in DRG neurons during colitis and in DRG culture

In colitis, it was determined through immunohistochemistry that DRG had an increased ( $p < 0.05$ ) protein expression of CGRP and this inflation was attenuated significantly ( $p < 0.05$ ) by treatment with anti-BDNF (Figure 7). In order to investigate whether anti-BDNF affects CGRP mRNA expression, RT-PCR was performed on three-day colitis-induced DRG. mRNA analysis after three-days induction was chosen over seven-days in order to accurately assess expression before protein translation and it was reported previously that at this time point CGRP mRNA was increased in the DRG [62]. It was determined that anti-BDNF reduced heightened CGRP mRNA transcription due to colitis (Figure 8). Further exploration of the role of BDNF on CGRP expression was conducted in culture through incubation of DRG explants with BDNF. Immunohistochemical analysis determined that BDNF significantly increased ( $p < 0.05$ ) CGRP protein expression *in vitro* (Figure 9).

### 4.2 Signaling pathways in BDNF-induced CGRP expression in culture

To examine the signaling pathways through which BDNF leads to CGRP expression, DRG explants treated with BDNF were also treated with signal cascade inhibitors. Immunohistochemistry showed that *in vitro* treatment of DRG with PLC inhibitor, U73122, decreased ( $p < 0.05$ ) BDNF-induced CGRP protein accumulation (Figure 10). MEK kinase inhibitor, PD98059, and PI3K inhibitor, LY294002 showed no significant changes in CGRP

expression *in vitro* (Figures 11 and 12). This suggests that BDNF-induced CGRP expression in DRG is mediated by the PLC- $\gamma$ , but not by the PI3K/Akt or the MEK (ERK) pathway in culture.

#### **4.3 PLC- $\gamma$ mediates colitis-induced CGRP expression in DRG**

Following *in vitro* inhibitor treatment, in order to determine the role of PLC- $\gamma$ , immunohistochemical analysis showed an increase ( $p < 0.05$ ) of PLC- $\gamma$  protein in colitis-induced DRG (Figure 13). In order to ascertain whether CGRP protein and PLC- $\gamma$  are related in response to BDNF treatment, co-localization was performed and it was demonstrated that the two proteins co-localized in small-diameter sensory neurons (Figure 14). To further investigate the role of PLC- $\gamma$  on CGRP expression, colitis-induced animals were treated with U73122 *in vivo*. Results showed that U73122 significantly reduced ( $p < 0.05$ ) CGRP expression in sensory neurons of DRG during colitis (Figure 15). Analysis of PLC- $\gamma$  levels in colitis-induced DRG after treatment with anti-BDNF was conducted to inspect the upstream role of BDNF on PLC- $\gamma$  activation. Anti-BDNF significantly reduced ( $P < 0.05$ ) levels of PLC- $\gamma$  in DRG sensory neurons during colitis (Figure 16).

#### **4.4 The PI3K/Akt pathway is not activated in colitis-induced CGRP expression in DRG**

In order to clearly distinguish the role of PI3K/Akt pathway from PLC- $\gamma$  pathway in DRG, lack of co-localization of Akt and CGRP determined that PI3K/Akt pathway was not involved in signal transduction to CGRP gene expression (Figure 17). Furthermore, *in vivo* treatment of LY294002 showed no significant change in CGRP protein expression (Figure 18).

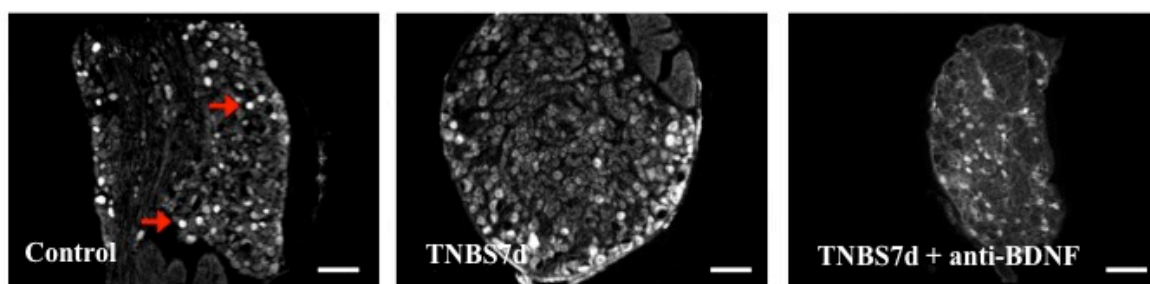
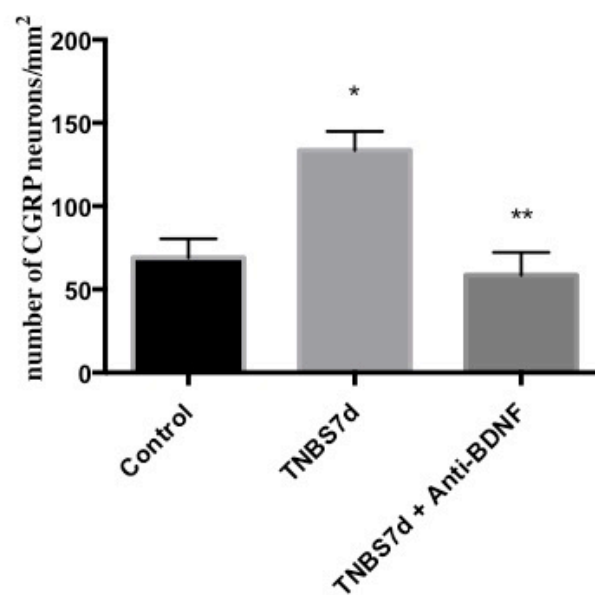
#### **4.5 Association of CREB activation with CGRP expression in DRG**

Co-localization was performed in order to determine if this transcription factor is associated with colitis-induced increase in CGRP protein expression. It was determined that activated CREB (pCREB) indeed co-localized with CGRP in small diameter DRG sensory neurons during colitis (Figure 19).

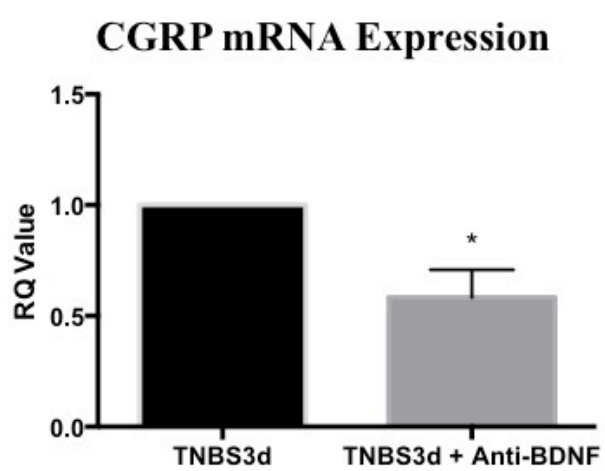
#### **4.6 CREB phosphorylation in DRG is regulated by endogenous and exogenous BDNF through the PLC- $\gamma$ pathway**

To further investigate the phosphorylation of CREB during colitis, immunohistochemistry was performed on colitis-induced DRG for pCREB. pCREB significantly increased ( $p < 0.05$ ) in TNBS7d DRG and this heightened response was reduced ( $p < 0.05$ ) greatly by anti-BDNF (Figure 20). Further examination of the role of pCREB in downstream BDNF signaling was determined through exogenous BDNF treatment of DRG explants. Western blot analysis of time course revealed that pCREB increased ( $p < 0.05$ ) significantly after five and 10 minute BDNF treatment (Figure 21). In order to investigate the signaling pathways involved in upstream of CREB activation, DRG explants were treated with LY294002, PD98059, and U73122. Through western blot, it was shown that U73122 decreased BDNF-induced CREB activation, whereas LY294002 and PD98059 showed no significant change in CREB phosphorylation (Figure 22).

**Figure 7. Changes in CGRP protein expression during colitis.** **A.** Immunohistochemistry displayed positively-stained CGRP cells in small diameter neurons. Arrows indicate positive staining. **B.** Analysis revealed that there is significant increase in CGRP protein expression in colitis-induced TNBS7d rats. Anti-BDNF decreased this heightened expression drastically. Bar = 50µm. \*, \*\*,  $p < 0.05$ . Results were obtained from 3 animals for each experimental group.

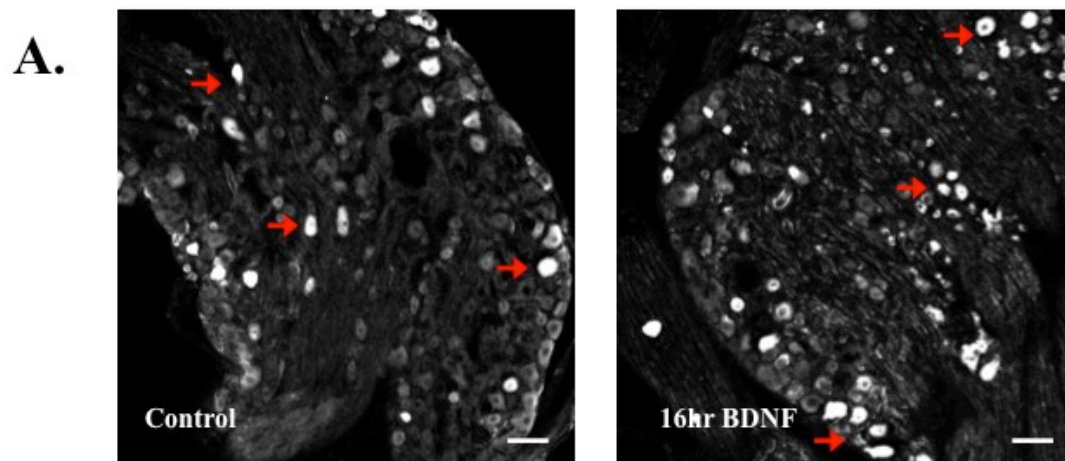
**A.****B. CGRP Protein Expression**

**Figure 8. Changes in CGRP mRNA expression during colitis.** CGRP mRNA levels were analyzed using RT-PCR. Treatment of rat DRG with anti-BDNF significantly decreased TNBS-induced inflammation in CGRP mRNA. \*,  $p < 0.05$ . Results were obtained from 3 animals for each experimental group

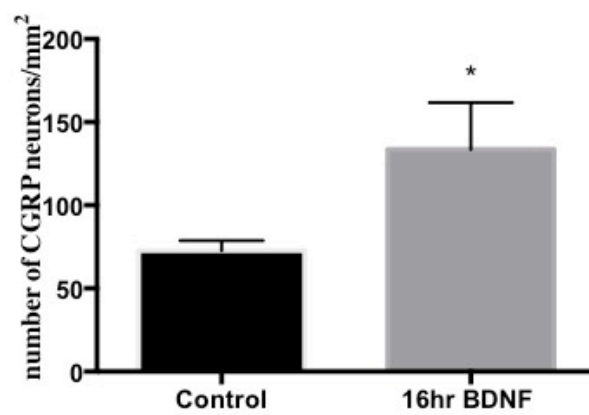


**Figure 9. Effects of BDNF on CGRP protein expression *in vitro*.** **A.** CGRP immunoreactivity in response to 16-hour treatment of 50ng/ml of BDNF on DRG explants. Arrows indicate positive staining. **B.** Evaluation of results revealed incubation with BDNF increased neurons positive for CGRP protein. Bar = 50 $\mu$ m. \*,  $p < 0.05$ . Results were obtained from 3 animals for each experimental group.

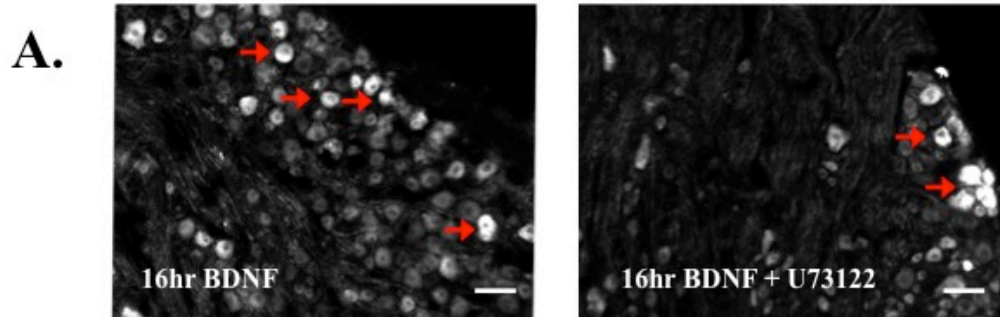




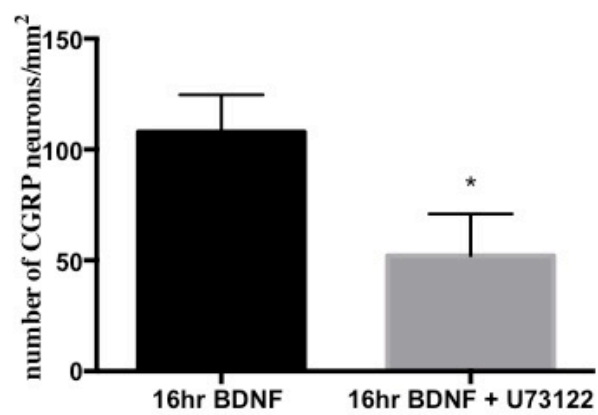
**B. CGRP Protein Expression**



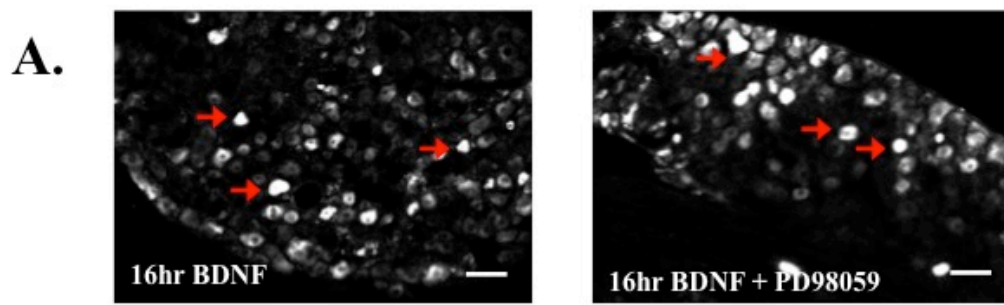
**Figure 10. Effects of PLC inhibitor on CGRP protein expression *in vitro*.** **A.** CGRP staining in response to BDNF treatment with addition of PLC- $\gamma$  inhibitor, U73122, in pair-matched DRG. Arrows indicate positive staining. **B.** In this *in vitro* treatment, there was a significant decrease in the number of neurons positive for CGRP. Bar = 25 $\mu$ m. \*,  $p < 0.05$ . Results were obtained from 3 animals for each experimental group.



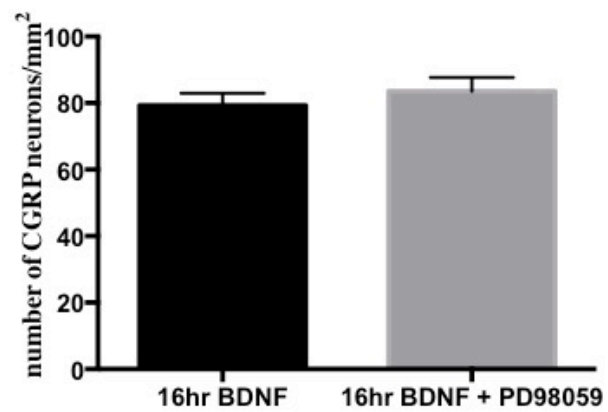
**B. CGRP Protein Expression**



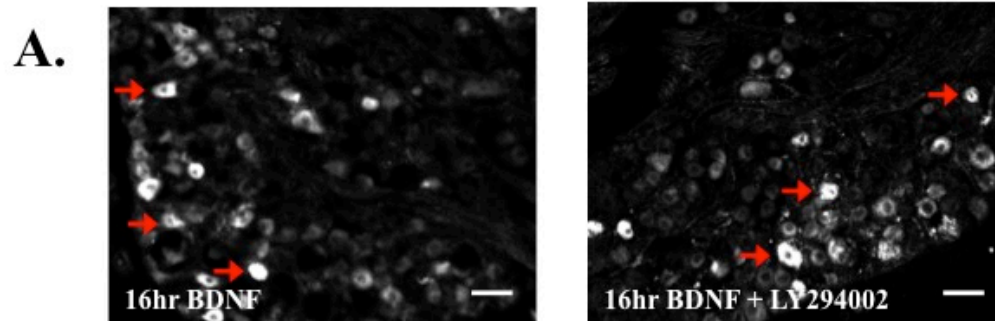
**Figure 11. Effects of MEK kinase inhibitor on CGRP protein expression *in vitro*.** **A.** CGRP immunoreactivity in response to 50ng/ml of BDNF with addition of MEK kinase inhibitor, PD98059, in pair-matched DRG. Arrows indicate positive staining. **B.** Analysis showed no significant change in the number of neurons positive for CGRP. Bar = 25µm. Results were obtained from 3 animals for each experimental group.



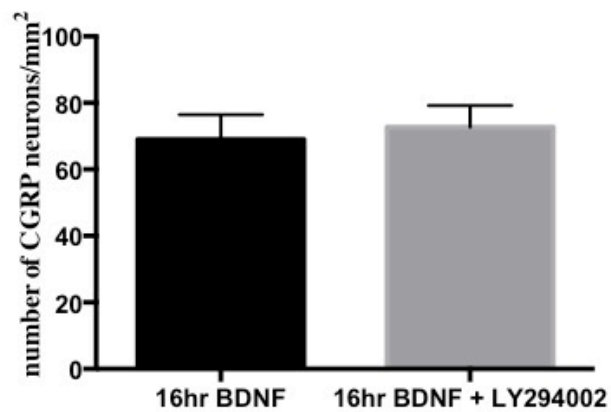
**B. CGRP Protein Expression**



**Figure 12. Effects of PI3K inhibitor on CGRP protein expression *in vitro*.** **A.** CGRP immunoreactivity in response to BDNF treatment with addition of PI3K inhibitor, LY294002, in pair-matched DRG. Arrows indicate positive staining. **B.** Results showed no significant change in cells positive for CGRP. Bar = 25µm. Results were obtained from 3 animals for each experimental group.

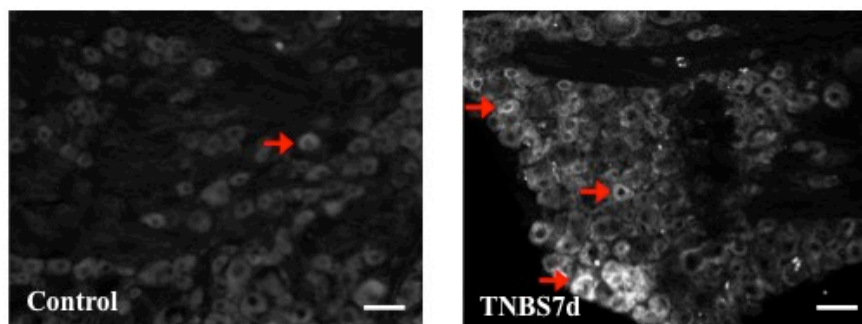
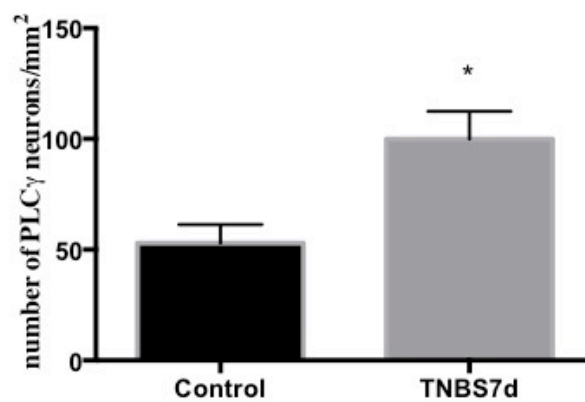


**B. CGRP Protein Expression**

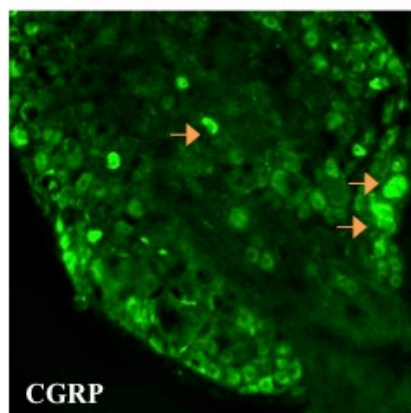
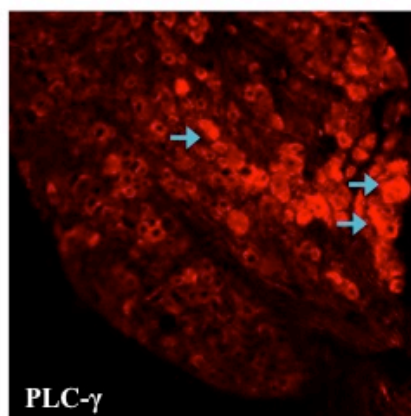
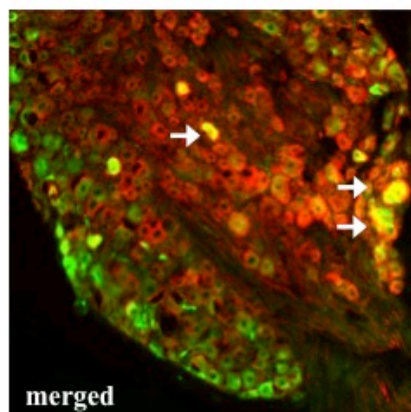


**Figure 13. PLC- $\gamma$  expression during colitis in rat DRG.** **A.** Shown are neurons positive for PLC- $\gamma$  in rat DRG treated with TNBS for seven days. Arrows indicate positive staining. **B.** Analysis determined that neurons positive for PLC- $\gamma$  significantly increased in DRG of colitis-induced rats. Bar = 50 $\mu$ m. \*,  $p < 0.05$ . Results were obtained from 3 animals for each experimental group

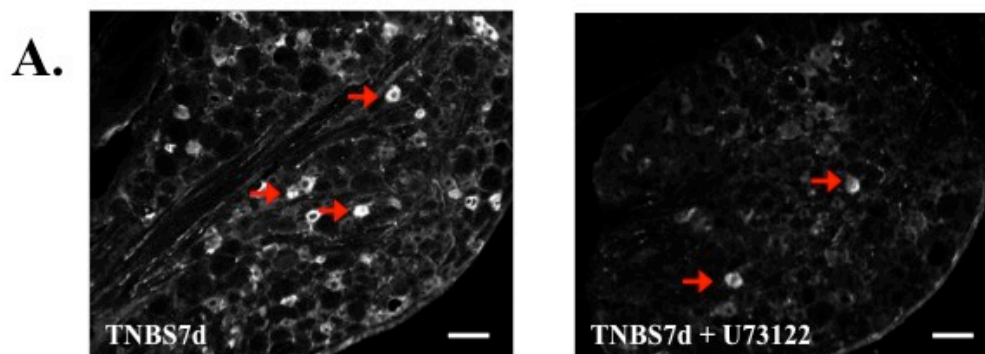


**A.****B. PLC- $\gamma$  Expression**

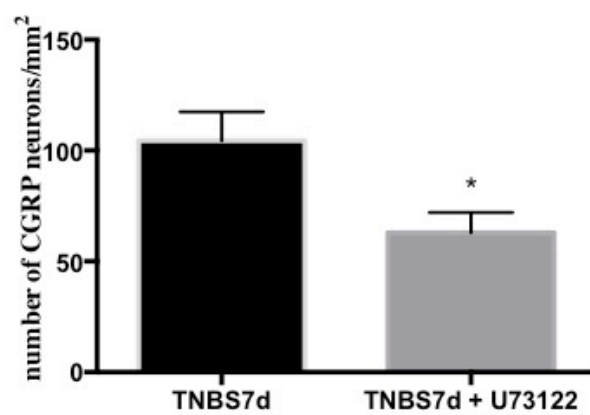
**Figure 14. Co-localization of CGRP and PLC- $\gamma$ .** Co-localization immunohistochemistry of PLC- $\gamma$  and CGRP in L1 rat DRG following *in vitro* incubation with 50ng/ml of BDNF. **A.** CGRP positively stained cells in DRG following incubation. **B.** PLC- $\gamma$  positively stained neurons are also present in the DRG. **C.** Co-localization (shown by arrows) revealed that CGRP co-localizes with PLC- $\gamma$ .

**A.****B.****C.**

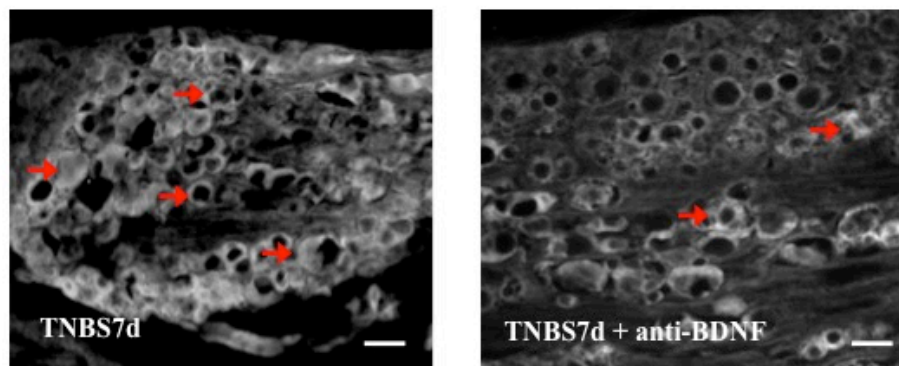
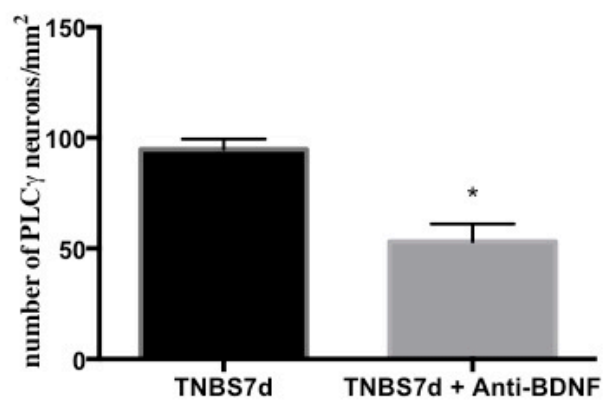
**Figure 15. PLC inhibitor treatment on CGRP protein expression in colitis.** **A.** CGRP immunoreactivity in response to *in vivo* PLC inhibitor treatment with a dose of 1-mg/kg body weight in colitis-induced rats. Arrows indicate positive staining. **B.** Analysis demonstrated significant decrease in positively stained neurons for CGRP. Bar = 50µm. \*,  $p < 0.05$  Results were obtained from 3 animals for each experimental group.



**B. CGRP Protein Expression**

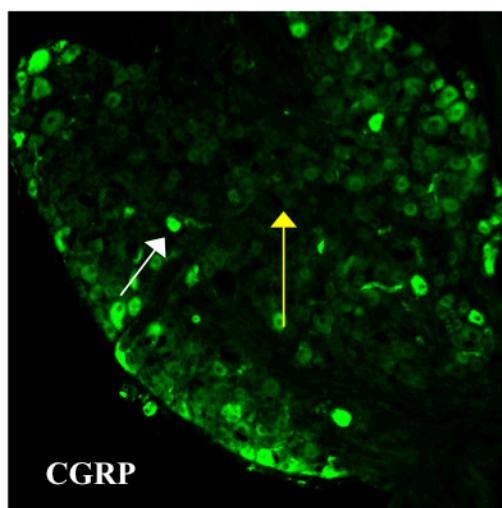
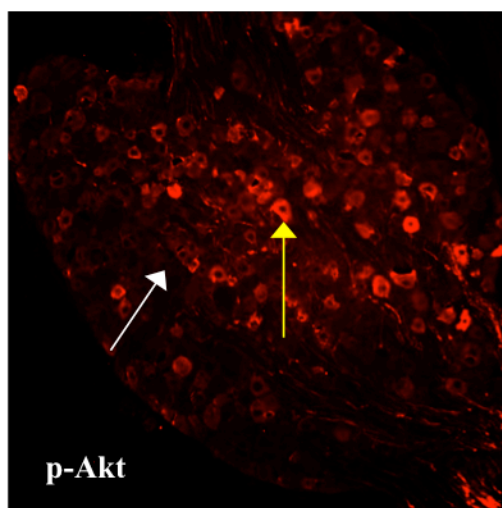
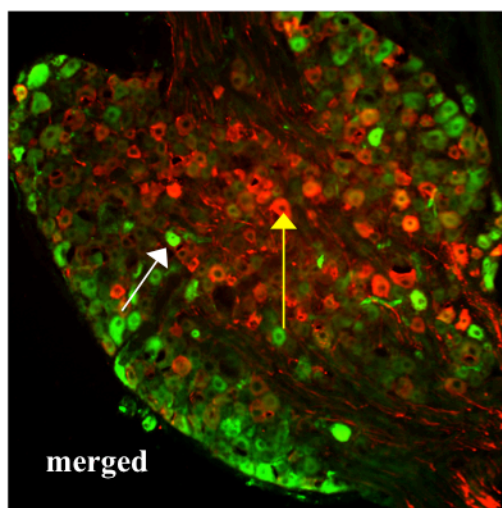


**Figure 16. PLC- $\gamma$  during anti-BDNF treatment in colitis-induced rat DRG.** **A.** Shown are neurons positive for PLC- $\gamma$  in rats treated with TNBS for seven days along with cells in response to co-treatment of TNBS7d and anti-BDNF. Arrows indicate positive staining. **B.** Results demonstrate neurons positive for PLC- $\gamma$  significantly decreased in DRG of colitis-induced rats treated with anti-BDNF. Bar = 50 $\mu$ m. \*,  $p < 0.05$  Results were obtained from 3 animals for each experimental group.

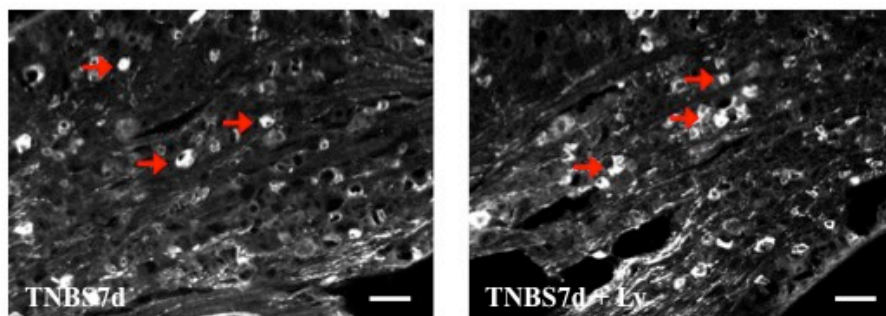
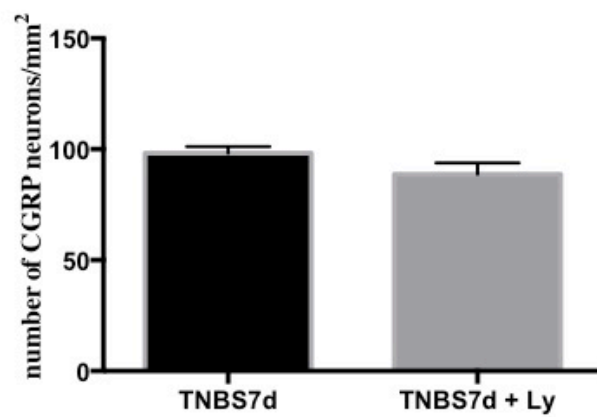
**A.****B. PLC- $\gamma$  Expression**

**Figure 17. Co-localization of CGRP and p-Akt.** Co-localization immunohistochemistry of p-Akt and CGRP in L1 rat DRG following *in vitro* incubation with 50ng/ml BDNF. **A.** CGRP positively stained cells in DRG following incubation. **B.** p-Akt positively stained cells are also present. **C.** Co-localization (shown by arrows) revealed that CGRP did not co-localize with p-Akt.

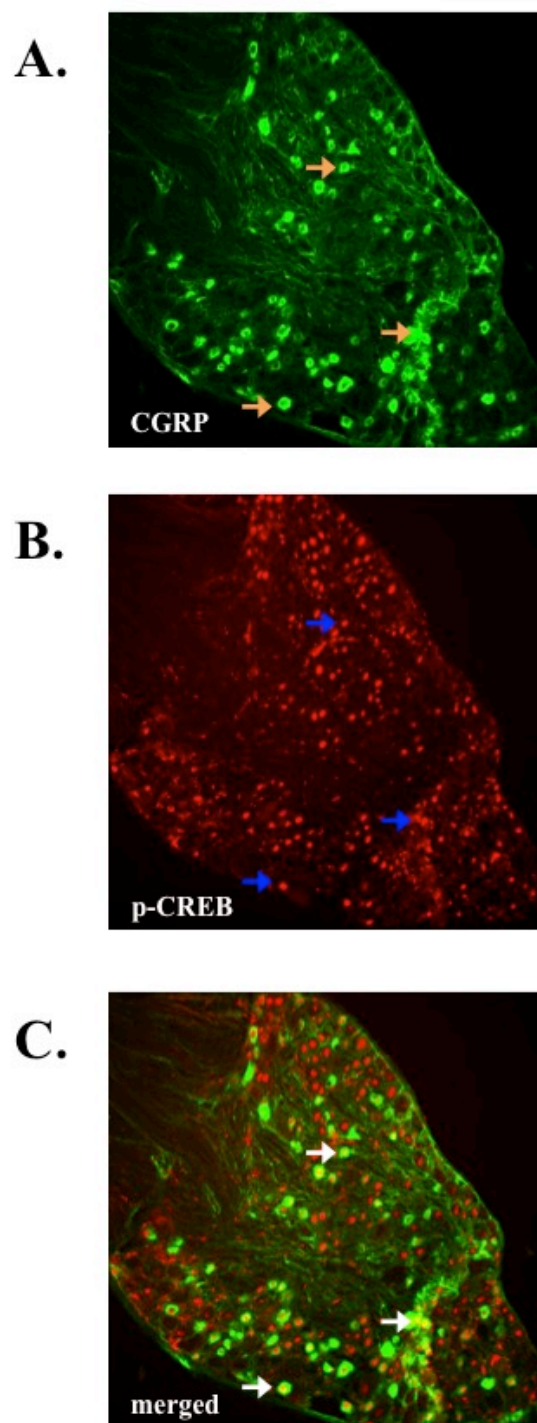


**A.****B.****C.**

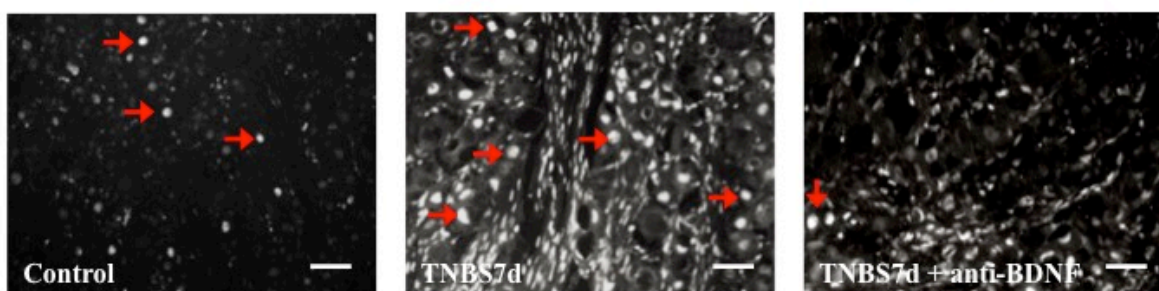
**Figure 18. PI3K inhibitor treatment on CGRP protein expression in colitis.** **A.** Neurons stained for CGRP in response to *in vivo* PI3K inhibitor treatment (dose of 50 µg/kg body weight) in TNBS7d rats. Arrows indicate positive staining. **B.** Results showed no significant change in neurons positive for CGRP. Bar = 50µ. Results were obtained from 3 animals for each experimental group.

**A.****B. CGRP Protein Expression**

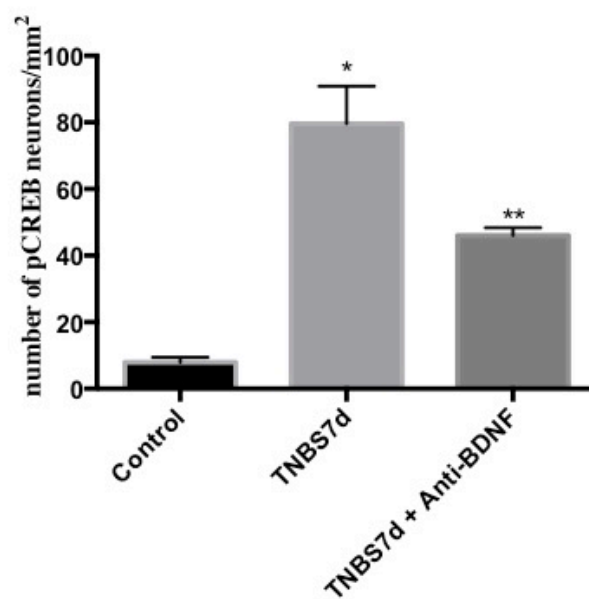
**Figure 19. Co-localization of CGRP and p-CREB.** Co-localization immunohistochemistry of p-CREB and CGRP in L1 rat DRG following dissection from colitis-induced TNBS7d rats. **A.** CGRP positively stained cells in DRG following incubation. **B.** p-CREB positively stained neurons are also present in the DRG. **C.** Co-localization (shown by arrows) demonstrates that CGRP co-localizes with p-CREB.



**Figure 20. CREB phosphorylation during colitis.** **A.** Immunohistochemistry displayed positively-stained p-CREB cells in small diameter neurons. Arrows indicate positive staining. **B.** Analysis revealed that there is significant increase in CREB phosphorylation in colitis-induced TNBS7d rats. Anti-BDNF decreased this upsurge significantly. Bar = 50 $\mu$ m. \*, \*\*,  $p < 0.05$ . Results were obtained from 3 animals for each experimental group.

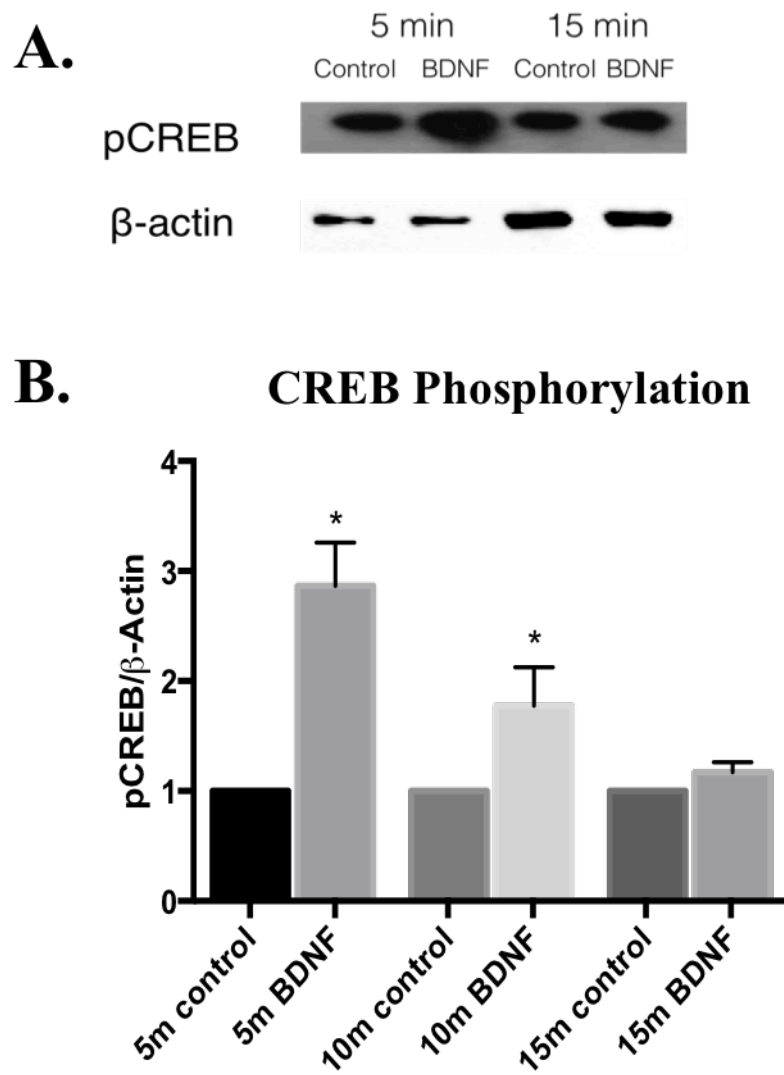
**A.****B.**

### CREB Phosphorylation

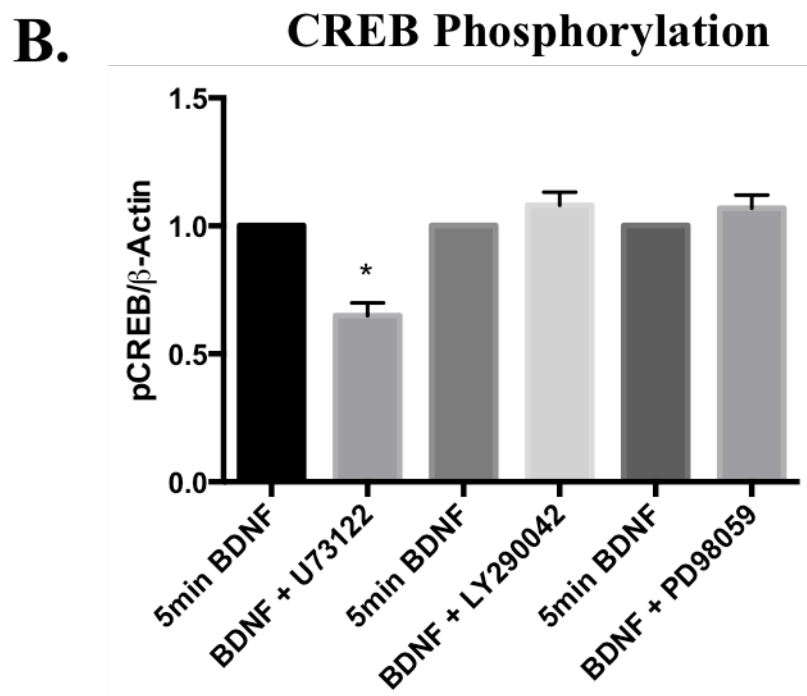
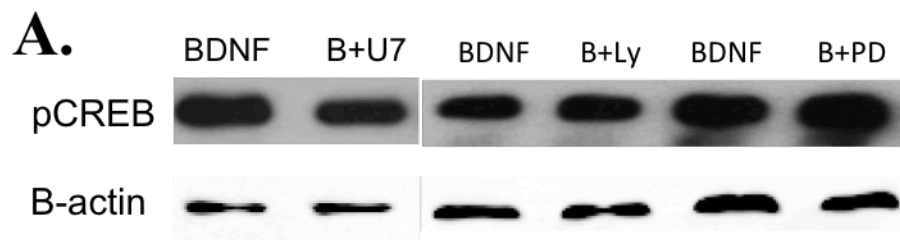


**Figure 21. Variations in CREB phosphorylation in DRG explants treated with BDNF A. and B.** Western blot analysis of p-CREB determined that BDNF increases CREB phosphorylation significantly after five and ten minute incubation. \*,  $p<0.05$ . Results were obtained from 3 animals for each experimental group.





**Figure 22. Upstream regulators of CREB phosphorylation *in vitro*.** Western blot of pair-matched DRG treated five minutes with BDNF and a signaling pathway inhibitor. U73122 decreased p-CREB levels, whereas LY294002 and PD98059 showed no change. \*,  $p < 0.05$ . Results were obtained from 3 animals for each experimental group



## Discussion

Inflammatory bowel disease is a chronic debilitating disease that causes inflammation and pain. This pain can be characterized by visceral hypersensitivity that increases sensitization of primary afferent sensory neurons to inflammatory signaling molecules and induces nociceptive transmission. In order to understand this mechanism of disease better, it is important to describe and outline the molecular mediators and their signaling pathways. The aim of this study was to identify the regulatory effects of BDNF on CREB phosphorylation and CGRP expression. CGRP and CREB are both signaling molecules that have been well identified to accompany nociceptive pain transmission. (64-70)

In this study, colitis caused an increase in the levels of CGRP and pCREB in sensory neurons of DRG, which was attenuated by anti-BDNF and PLC inhibitor, U73122, but not PI3K inhibitor, LY294002. BDNF-induced CGRP expression and CREB activation in DRG culture was also blocked by PLC inhibitor, U73122, but not PI3K inhibitor, LY294002, or MEK kinase inhibitor, PD98059. This role of PLC- $\gamma$  is further confirmed by the co-localization of CGRP with PLC- $\gamma$ , but not phosph-Akt. CGRP and pCREB co-localization indicate the regulatory role CREB transcription factor may play on CGRP mRNA transcription. These results suggest that the PLC- $\gamma$  pathway, a unique pathway in signal transduction, is mediating BDNF action on sensory neuronal activation during colitis

Previous studies have shown that experimental animals induced with colitis show visceral hyperalgesia [62]. In these TNBS-induced animals, CGRP mRNA transcripts increase. For this study, TNBS animals were also treated with anti-BDNF and this resulted in an attenuation of CGRP mRNA. Increased CGRP protein expression also was attenuated with anti-BDNF. Previous studies demonstrated that BDNF increased in response to inflammation [71] and the results from this suggest that BDNF plays an important role in expression of the important nociceptive pain marker, CGRP. To examine the role of BDNF in CGRP protein expression more carefully, lumbosacral DRG were explanted from rat spinal cord and incubated with BDNF. Immunohistochemical analysis revealed the CGRP protein levels increased, demonstrating the BDNF-CGRP link with *in vitro* studies. BDNF thus is an important regulator in CGRP protein expression and sensory pain transmission. It has been demonstrated that there is a NGF-CGRP signaling axis and analysis of the results presented show a parallel signaling axis between BDNF and CGRP. It has been shown that NGF can increase CGRP and that NGF also is able to increase BDNF in DRG. This relationship with current results implies that NGF can increase CGRP through its own receptor, TrkA, or NGF can mediate BDNF regulation upregulation, which can induce CGRP expression through its receptor TrkB.

BDNF knockout mice have previously shown to have lower mucosal nerve fiber density and also lower visceral hypersensitivity to colonic distension [72]. Similarly, CGRP knockout mice are unable to induce visceral hypersensitivity [51]. Attenuation of CGRP and pCREB with anti-BDNF is consistent with these studies and provides a

mechanistic exploration underlying BDNF's role in synaptic plasticity and remodeling during visceral inflammation.

Furthermore, to determine the signaling mechanism behind these regulatory changes, explants were treated with various signaling pathway inhibitors as well as BDNF to determine if inhibition of a signaling molecule led to attenuation of BDNF effects on CGRP protein. Inhibition of the PLC- $\gamma$  pathway, but not the PI3K/Akt or MAPK pathways, led to decreased CGRP protein expression and CREB activation both *in vivo* and *in vitro*. CGRP also co-localizes with PLC- $\gamma$  and CREB in sensory neuron cells of L1 DRG, whereas p-Akt did not positively co-localize with CGRP. These results suggest a novel mechanism through which BDNF influences sensory neuron activation. Studies have shown that BDNF-up-regulation is triggered by increase in NGF signaling through the PI3K/Akt pathway [71]. However, results suggest that BDNF itself works to activate CREB through the PLC- $\gamma$  pathway. PLC- $\gamma$  can be activated by receptor tyrosine kinase phosphorylation and can also regulate  $\text{Ca}^{2+}$  entry that may affect the activation of various transcription factors, such as CREB. This pathway is crucial in neuronal plasticity in CNS signaling and LTP. The role of the PLC- $\gamma$ / $\text{Ca}^{2+}$  in visceral hypersensitivity has not been explored and this finding suggests the PLC- $\gamma$  pathway is critical in peripheral neuronal plasticity in response to visceral inflammation.

The PLC- $\gamma$  pathway may regulate CREB in this signaling axis and to further investigate the intrinsic mechanism, levels of pCREB were observed in relationship to BDNF. BDNF increased pCREB protein *in vitro* and in colitis. Through *in vitro* treatment of DRG with PLC inhibitor, it was discovered that the treatment decreased activation of

CREB. MEK kinase inhibitor and PI3K inhibitors showed no change. The PLC- $\gamma$ -mediated CREB phosphorylation can be triggered by PLC- $\gamma$ -modulated  $\text{Ca}^{2+}$  release or influx and further activation of  $\text{Ca}^{2+}$  dependent kinase activation, such as Calcium/calmodulin-dependent protein kinase II (CaMKII). PLC- $\gamma$  can induce this  $\text{Ca}^{2+}$  increase through cleavage of phospholipid phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ).  $\text{IP}_3$  can then bind to  $\text{IP}_3$  receptors, namely  $\text{Ca}^{2+}$  channels in the smooth endoplasmic reticulum. DAG functions to activate PKC, which in turn activates other molecules that alter cellular activity. Therefore, PLC- $\gamma$  pathway is a novel mechanism in response to visceral inflammation that modulates many secondary messengers in activation of a number of transcription factors, one possibility being CREB.

In conclusion, these results demonstrate that there is a BDNF-CREB-CGRP signaling axis that accompanies inflammation and plays a role in visceral hypersensitivity. The regulation of CREB and CGRP by BDNF leads to nociceptive pain transmission through activation of CREB, alteration in gene transcription and protein synthesis, and delivery of transmitter neuropeptides to nerve terminals.

### List of References



### List of References

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